



Original article

Improvement of antioxidant activity of oregano (*Origanum vulgare* L.) with an oral pharmaceutical formMaría Pilar de Torre^a, Jose Luis Vizmanos^{b,c}, Rita Yolanda Caverio^{b,d}, María Isabel Calvo^{a,b,*}^a Department of Pharmaceutical Technology and Chemistry, School of Pharmacy and Nutrition, University of Navarra, Irúnlarrea 1, 31008 Pamplona, Spain^b IDISNA-Instituto de Investigación Biosanitaria de Navarra, 31008 Pamplona, Spain^c Department of Biochemistry & Genetics, School of Sciences, University of Navarra, Irúnlarrea 1, 31008 Pamplona, Spain^d Department of Environmental Biology, School of Sciences, University of Navarra, Irúnlarrea 1, 31008 Pamplona, Spain

ARTICLE INFO

Keywords:

C. elegans
Antioxidant
O. vulgare
Formulation
Digestion

ABSTRACT

Aging-related diseases can be triggered by multiple factors such as oxidative stress. Oxidative stress is an imbalance between free radicals and antioxidants, so today, compounds capable of reducing or neutralizing free radicals are being studied for a therapeutic use. *Origanum vulgare* L. is a traditional medicinal plant used for a wide number of health problems due to its antimicrobial, carminative and antioxidant activities. However, when administered orally, gastrointestinal digestion can modify some of therapeutic properties. To avoid this, two different solid oral formulations have been designed for an *O. vulgare* extract evaluating their antioxidant behaviours *in vitro* and *in vivo* after a simulation of gastrointestinal digestion. The results showed that the divided powder has a lower antioxidant activity both *in vitro* and *in vivo* than the encapsulated extract. The quantitative difference of polyphenols found on HPLC-DAD (especially luteolin, apigenin and caffeic acid) may explain the differences in pharmacological activity. Thus, we propose that the best form to administrate *O. vulgare* extracts to maintain the antioxidant properties is the encapsulated form, that is, two capsules of 250 mg of a hydroalcoholic extract of *O. vulgare* with a minimum of 33 % of rosmarinic acid as a daily dose.

1. Introduction

Origanum vulgare L. has traditionally been used in food industry and cooking as a condiment, but also as a digestive and circulatory stimulant. The essential oil from this plant is also used in aromatherapy and perfumery, including soaps. Some other traditional uses include the preparation as an infusion to treat digestive disorders, headaches, sore throats or colds due to the antispasmodic, calnative, carminative, diaphoretic, expectorant, stomachic and tonic effects [1]. Additionally, most of the components of *O. vulgare* have been shown to have an antioxidant activity *in vitro* by reducing free radicals formation [2–5] and their antioxidant and cytoprotective effects *in vivo* [6–8]. The accumulation of free radicals in the body has been considered one of the factors involved in the development of various diseases related to aging, so the identification of compounds capable of reducing them are of interest.

The route of administration of a drug plays an important role in its pharmacological activity. In general, oral administration which is

widely used is preferred because of its comfort for the patient. In the specific case of the medicinal plants, the particle size of the extracts can be very heterogeneous and have several chemical compounds responsible for their action that can be modified by gastrointestinal digestion (which involves enzymes and pH variation). For these reasons, the pharmaceutical formulation for oral administration can be very important in the pharmacokinetics and pharmacodynamics of these products [9]. In the laboratory, it is possible to simulate the effects of the gastrointestinal digestion process on the chemical structure of the different components of the extracts using an *in vitro* model and then analyze their final pharmacological activity both *in vitro* and *in vivo*. Among the different *in vitro* methods to quantify antioxidant activity, the analysis of the scavenging activity of DPPH[•] radical is the most frequently used method [10]. On the other hand, *Caenorhabditis elegans* is a good *in vivo* model widely used due to its simplicity, low price, reproducibility and molecular similarity with humans [11]. There are several assays already described to study the anti-aging and antioxidant effect produced by a compound in this organism, one of them recently

Abbreviations: OV-E, Crude extract; OV-C, Intestinal absorbable fraction of encapsulated extract; OV-P, Intestinal absorbable fraction of powder extract

* Corresponding author at: Department of Pharmaceutical Technology and Chemistry, School of Pharmacy and Nutrition, University of Navarra, Irúnlarrea 1, 31008 Pamplona, Spain.

E-mail addresses: mdetorre@alumni.unav.es (M.P. de Torre), jlvizmanos@unav.es (J.L. Vizmanos), rcavero@unav.es (R.Y. Caverio), mcalvo@unav.es (M.I. Calvo).

<https://doi.org/10.1016/j.bioph.2020.110424>

Received 13 May 2020; Received in revised form 26 May 2020; Accepted 13 June 2020

Available online 19 June 2020

0753-3322/ © 2020 Published by Elsevier Masson SAS. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

published by our group [12].

The aim of this report is to determine the antioxidant activity of two different oral pharmaceutical forms of *O. vulgare* hydroalcoholic extracts using both *in vivo* and *in vitro* models, before and after an *in vitro* simulation of the gastrointestinal digestion process.

The bioaccessibility, defined by the amount of compound available for absorption, can be conditioned by several factors. Some of these factors are very influenced by the chemical structure (glycosylation, esterification and polymerisation) [13,14]. Pharmaceutical formulation can be used to preserve the chemical structure that could be modified along the digestive tract. Although sometimes this effect can be circumvented by the administration of pro-drugs [15], plant extracts are made up of multiple compounds so pharmaceutical formulation is a good physical solution to prevent their degradation. Thus, two simple oral formulations were designed. The first one is a divided powder with a previous lyophilization to improve the stability of its components. This powder, mixed with water, is the simplest form for oral administration. The second one is a hard gelatine capsule that contains the extract. The capsule acts as a physical protective barrier of the chemical compounds responsible for pharmacological activity. We have evaluated the influence of both formulations on bioaccessibility and pharmacological activity (both *in vitro* and *in vivo*) after a simulated process of gastrointestinal digestion and we have also performed a chemical characterization by HPLC-UV to determine the changes produced in the composition of the plant extracts throughout the process. These data will allow to choose the form with the best bioaccessibility.

2. Materials and methods

2.1. Plant material, extraction and pharmaceutical form preparation

Flowered aerial parts from *Origanum vulgare* L. were collected in June 2017 in Santacara (Navarra, Spain). A voucher specimen (PAMP21629) was deposited in the herbarium of the Department of Environmental Biology at the School of Sciences, University of Navarra (Pamplona, Spain) after its authentication by Dra. R. Y. Caverio.

The hydroalcoholic extract was prepared from flowered aerial-dried parts (10 g) that were macerated at 4 °C in 250 mL of ethanol-water 50 % v/v for 24 h and then filtered by gravity. Maceration process was repeated four times to extract as many components as possible and then concentrated using a rotary-evaporator. Finally, the dry hydroalcoholic extract (final yield of 3:1) was lyophilized and stored at - 40 °C in a freezer until use.

Then, we select two simple solid oral pharmaceutical forms for this extract, divided powder and hard gelatine capsules. The divided powder was composed by extract of *O. vulgare* (with at least 30 % of rosmarinic acid) and antiagglomerant (silicon dioxide E551). A daily dose of 500 mg of this form dissolved in water once a day would provide a minimum of 170 mg of rosmarinic acid. The gelatine capsule was composed by extract of *O. vulgare* (with at least 30 % of rosmarinic acid) and antiagglomerant (silicon dioxide E551) into a gelatine capsule n° 2. A daily dose of 500 mg of extract would consist of two capsules once a day, which would also provide a minimum of 170 mg of rosmarinic acid.

Quality control assays were performed in a small scale in three replicates as a control in the production for each oral preparation. According to Pharmacopeia (Real Farmacopea Española, 5th Edition), capsules should satisfy uniformity content (assay B, 2.9.6), uniformity mass (2.9.5) and disintegration assay (2.9.1), whereas divided powder only needs to satisfy uniformity content and mass content for single-dose powder preparations.

2.2. *In vitro* gastrointestinal digestion process

The *in vitro* gastrointestinal digestion process was performed according to Gayoso et al. (2018) with some modifications. Enzymatic

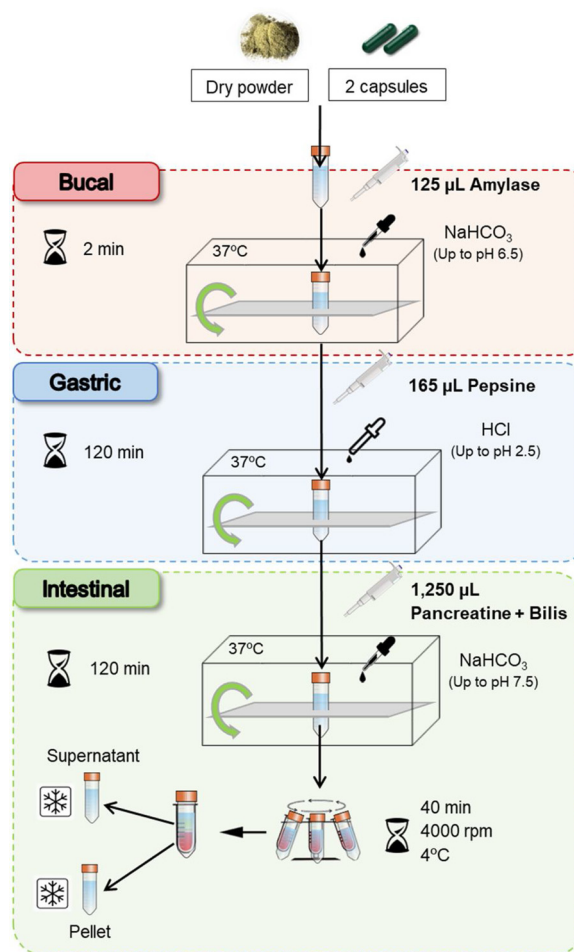


Fig. 1. Diagram of the *in vitro* gastrointestinal digestion process with the three main steps (bucal, gastric and intestinal). Final samples after centrifugation were the intestinal absorbable fraction supernatant (supernatant after final centrifugation) and the intestinal non-absorbable fraction (pellet after final centrifugation).

solutions were freshly prepared and kept on ice until use: α -amylase (#A1031, Sigma-Aldrich Co., St. Louis, MO) 1.3 mg/mL in 1 mM CaCl_2 ; pepsin (#77161, Sigma-Aldrich Co., St. Louis, MO) 160 mg/mL in 0.1 M HCl; bile (#B8631, Sigma-Aldrich Co., St. Louis, MO) 25 mg/mL plus 0.1 g of pancreatin (#P7545, Sigma-Aldrich Co., St. Louis, MO) in 0.1 M NaHCO_3 . Daily dose of each pharmaceutical form (equivalent a 500 mg of extract) were mixed with 12.5 mL of water and the digestion process was performed in 50-mL tubes rotating at 22 rpm inside an incubator at 37 °C (Fig. 1). The oral phase (2 min) started after adding 125 μL of amylase solution and adjusting to physiological pH 6.5 with 1 M NaHCO_3 . Then, 165 μL of pepsin solution were added at pH 2.5 with 3 M HCl for the gastric step (2 h). Intestinal step (2 h) was simulated by adding 1250 μL of pancreatin-bile solution and adjusting pH to 7.5 with 1 M NaHCO_3 . After that, the intestinal mixture was centrifuged (at 51.070 g, 40 min, 4 °C) and the supernatant (absorbable fraction) was separated from the pellet (non-absorbable fraction). Finally, both fractions were lyophilized (Cryodos50, Telstar, Barcelona, Spain). A control with two empty capsules and a blank without plant extract were treated under the same conditions.

The bioaccessibility was expressed as a percentage by using the formula [16,17]:

$$\text{Bioaccessibility (\%)} = \frac{\text{final concentration} \left(\frac{\text{mg}}{\text{mL}} \right)}{\text{initial concentration} \left(\frac{\text{mg}}{\text{mL}} \right)} \times 100$$

in which the final concentration is the concentration of the mixture after digestion (taking into account the enzymes and the solutions added to adjust pH in each step) and the initial concentration is the concentration of the sample prepared before this process (40 mg/mL). The bioactivity, defined as the physiological response, was adjusted taking into account this bioaccessibility of the pharmaceutical form by applying the percentage obtained to the results of the antioxidant assays:

$$\text{Bioactivity} = EC_{50} \text{ antioxidant activity } \left(\frac{\mu\text{g}}{\text{mL}} \right) \times \text{Bioaccessibility of pharmaceutical form (\%)}$$

2.3. *In vitro* antioxidant activity assay

Antioxidant activity can be monitored using the scavenging effect of radicals on DPPH[•] (#D9132, Sigma-Aldrich Co., St. Louis, MO). This activity was firstly confirmed by using thin layer chromatography (TLC) as a qualitative assay disposing 10 µL of hydroalcoholic extract (crude extract) (10 mg/mL) in a Silicagel 60 F₂₅₄nm with plastic base (#105554, Merck KGaA, Darmstadt, Germany) that were eluted with ethyl acetate - acetic acid - formic acid - water (100:11:11:26) in a chromatography chamber. After separation, the plate was sprayed with a 0.4 mg/mL DPPH[•] solution to identify the antioxidant activity by turning into yellow.

The scavenging activity of the extract before and after digestion of the two formulations (capsule and powder) were also quantified using rosmarinic acid (#536954, Sigma-Aldrich Co., St. Louis, MO) as positive control. Lyophilised samples (before and after digestion) were dissolved in either water or ethanol - water (1:1 v/v) at 10 different serial concentrations (1000–1.95 µg/mL) [18]. The reaction was monitored every 15 min for 90 min, registering absorbance at 517 nm to calculate scavenging activity (% of inhibition) with the formula:

$$\text{Scavenging activity (\%)} = \left(1 - \frac{Abs_{\text{sample}} - Abs_{\text{blank}}}{Abs_{\text{control}} - Abs_{\text{blank}}} \right) \times 100$$

In which Abs_{sample} is the absorbance at 517 nm of the reaction in presence of sample (sample dilution + DPPH[•] solution), Abs_{blank} is the absorbance of the blank for each sample dilution (sample dilution + DPPH[•] solvent) and Abs_{control} is the absorbance of control reaction (sample solvent + DPPH[•] solution).

Values in each point were expressed as EC₅₀, that is the concentration in which the 50 % of the free radical DPPH[•] is reduced.

2.4. *In vivo* antioxidant activity assay

In vivo antioxidant activity of the hydroalcoholic crude extract and the two absorbable fractions obtained after the digestion process was quantified using the method recently described in *C. elegans* [12]. For both formulations, the absorbable fraction of rosmarinic acid was used as positive control and as a reference besides the non-digested extract. For the powder, we evaluated the blank with no extract as a negative control to verify the absence of activity of the residual enzymes. For the formulation in capsules, the lyophilised powder of the negative control of two empty capsules was also analysed. For all the samples, six different concentrations were tested (50, 20, 10, 1, 0.1 and 0 mg/mL) and the EC₅₀ value from the scavenging activity of DPPH[•] was plotted as described [12] using GraphPad Prism v6.01 (GraphPad Software, La Jolla, CA).

2.5. Chemical characterisation

O. vulgare hydroalcoholic extract chemical composition was firstly qualitatively analysed by TLC, using the same stationary and mobile phases suitable for flavonoids and phenolic compounds described

before [19]. Fractions obtained from the *in vitro* gastrointestinal process were also analysed. Spots were observed at visible light, 254 nm and 366 nm. A better visualisation of the spots was obtained after treatment with NP reagent (#126705, Sigma-Aldrich Co., St. Louis, MO) at 366 nm.

The main compounds of the extracts were quantitatively identified by High Performance Liquid Chromatography with Diode Array Detector (Waters HPLC 600E multi-solvent delivery system, a Waters U6K sampler and a Waters 991 photodiode-array detector, Waters Corp., Milford, MA). Samples were injected in a C18 reversed phase column (Nova-Pak 150 mm × 3.9 mm, 4 µm, Waters Corp., Milford, MA) at 25 °C with a flow rate of 0.8 mL/min and were eluted with acetonitrile (#34851, Sigma-Aldrich Co., St. Louis, MO) (solution A) and acidified water type I adjusted to pH 2 with formic acid (#33015, Sigma-Aldrich Co., St. Louis, MO) (solution B), in different proportions (%) of solution B: 0–10 min, 95 %; 10–20 min, 95–90 %; 20–35 min, 90–80%; 35–45 min, 80–60 %; 45–50 min, 80–20 % and then 95 % in 5 min. The range of detection was established between 210 and 550 nm.

Results were used as a reference for the extract and compared with the absorbable and non-absorbable fractions of the two formulations. Identification of compounds was carried out according to literature and the use of standard samples (caffeic acid -#C0625-, rosmarinic acid -#R4033-, apigenin -#10,798-, luteolin -#L9283-, luteolin-7-O-glucoside -#1,370,837-, and 3,4-dihydroxybenzoic acid # 37,580 -, all from Sigma-Aldrich Co., St. Louis, MO) at five different concentrations to build a calibration curve. Thus, the areas under the curve (AUC) of the main peaks were expressed in terms of mg of the standard compound per 100 g of extract by linear regression analysis. The reproducibility of the chromatographic separations was verified with a second HPLC process [20]. All samples were injected in triplicate.

2.6. Statistical analysis

EC₅₀ values were generated with GraphPad Prism, v6.01 (GraphPad Software, La Jolla, CA). Means, standard deviations and graphs were obtained with Microsoft Excel 2013 (Microsoft Corp., Redmond, WA). Statistical analysis was performed using Stata v.12 (StataCorp LLC, College Station, TX). Normality was checked by Shapiro-Wilkinson test. Differences were estimated by ANOVA followed by pairwise comparison post hoc test using Tukey's method (99.9 % CL) or post-estimation margins to check interaction among groups.

3. Results

3.1. Preparation of the pharmaceutical forms: quality control assays

The capsules must fulfil assay B of content uniformity (2.9.6) in the mean ± 15 %. Our formulation met these requirements, being the total mass 311.15 ± 0.94 mg for individual full-filled capsule with a content of 250.01 ± 0.64 mg. The uniformity mass assay (2.9.5) accepts a 10 % of deviation for capsules with less than 300 mg. This value in our formulation was a 0.03 %. Divided powder preparations also met content uniformity and mass assays, being their values in the range of 502.04 ± 0.06 mg.

The disintegration of the capsules during the *in vitro* gastrointestinal digestion process also satisfied the established conditions for capsules and tablets (2.9.1) (i.e., capsules were disintegrated after 30 min at gastric conditions in a solution with HCl 0.1 M or gastric enzymes). Empty capsules, whose result for uniformity and mass assay was 61.14 ± 0.01 mg/mL, also met such requirements.

3.2. *In vitro* gastrointestinal digestion process: bioaccessibility

The process of the *in vitro* gastrointestinal digestion involves the addition of enzymes and a pH adjustment according to the physiology

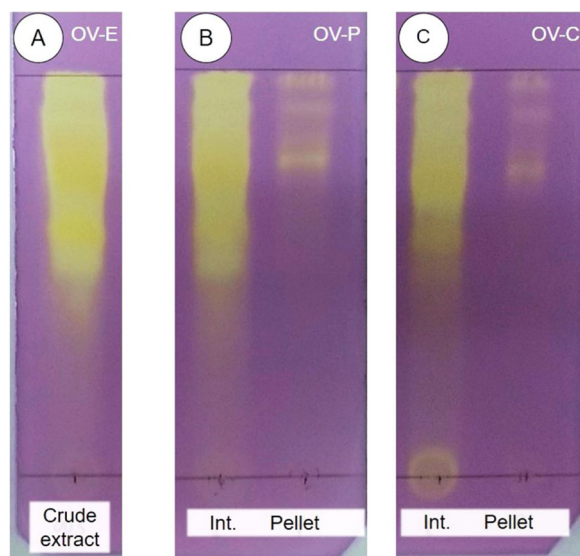


Fig. 2. Qualitative antioxidant assay on TLC. (A) OV-E: non-digested hydroalcoholic extract (crude extract). (B) OV-P: extract in powder after gastrointestinal digestion. (C) OV-C: extract in capsules after digestion. Int.: intestinal absorbable fraction; Pellet: intestinal non-absorbable fraction.

of the gastrointestinal tract at every stage. The effects of this process depend on the nature of the initial sample. The bioaccessibility of the two formulations (capsule and powder) was expressed as the percentage of the final concentration with respect to the initial concentration [12], being 87.17 % and 83.11 % for the encapsulated extract and the powder respectively.

3.3. Antioxidant activity assays

Qualitative TLC assay with DPPH[•] showed that the two formulations retained antioxidant activity after the gastrointestinal digestion process. The purple DPPH[•] solution turns into yellow in presence of antioxidants. Fig. 2 shows the results on TLC for the crude extract and both the intestinal absorbable and non-absorbable fractions (pellets) from the two formulations.

Antioxidant activity of the crude extract and the intestinal absorbable fractions from both formulations was then quantified also by DPPH[•] method, recording values of absorbance at 517 nm every 15 min for 90 min and calculating EC₅₀ values in each time point (Fig. 3). The activity of the non-absorbable fraction (pellet) was not quantified because this part will be excreted without being absorbed.

The reaction is stable when no statistical differences ($p > 0.05$) are observed between two consecutive values. For the positive control, the reaction with digested rosmarinic acid (RA) takes more time to stabilize than the reaction with the non-digested RA (before digestion: $p = 0.120$ between EC₅₀ at 15 min and EC₅₀ at 30 min; after digestion: $p = 0.991$ between EC₅₀ at 30 min and EC₅₀ at 45 min). The extract administrated as powder has a similar behaviour but delayed in time (before digestion: $p = 0.475$ between EC₅₀ at 30 min and EC₅₀ at 45 min; after digestion: $p = 0.999$ between EC₅₀ at 45 min and EC₅₀ at 60 min). However, the reaction of the extract encapsulated after digestion stabilizes at the same time as the non-digested extract ($p = 0.945$ between EC₅₀ at 30 min and EC₅₀ at 45 min).

Rosmarinic acid showed no statistical differences in scavenging activity before and after digestion *in vitro* at the time of stability of the reaction: EC₅₀, 15 min = $1.71 \pm 0.04 \mu\text{g/mL}$ and EC₅₀, 30 min = $1.88 \pm 0.15 \mu\text{g/mL}$, respectively with $p = 0.999$. However, the pharmaceutical form has influence in the antioxidant activity of the hydroalcoholic extract. The encapsulated hydroalcoholic extract showed no statistical differences before and after digestion at the time

of stability: EC₅₀, 30 min = $4.06 \pm 0.39 \mu\text{g/mL}$ and EC₅₀, 30 min = $3.77 \pm 0.30 \mu\text{g/mL}$, respectively with $p = 0.981$. By contrast, scavenging activity after digestion of the extract administrated as powder was significantly increased, which means a lower antioxidant activity ($p = 0.001$, EC₅₀, 45 min = $4.86 \pm 0.58 \mu\text{g/mL}$). In fact, both formulations show different antioxidant activities *in vitro* ($p < 0.001$).

The antioxidant activities of the samples and the positive control were statistical different at all time-points ($p < 0.001$). As expected, empty capsules digested (used as blank for encapsulated extract) and the Blank sample (digestion without sample) did not show any antioxidant activity *in vitro*. For further information, scavenging activity (as % inhibition) is compiled in supplementary table S1.

The antioxidant activity of the different extracts was also monitored *in vivo* after *C. elegans* treatment every 15 min for 90 min. Results expressed in EC₅₀ were represented also as a function of time (Fig. 4). In this case, rosmarinic acid reached stabilisation of the reaction earlier when the sample had previously been digested *in vitro* (before digestion: $p = 0.695$ between min 60 and 75; after digestion: $p = 0.631$ between min 45 and 60). Like the results obtained with *in vitro* assays, the extract showed a different behaviour in stabilisation depending on the pharmaceutical form. The extract administrated as a powder showed similar results to rosmarinic acid, reaching a stabilization earlier than the crude extract (before digestion: $p = 0.343$ between min 45 and 60; after digestion: $p = 0.825$ between min 30 and 45). In contrast, encapsulated extract required the same time as the non-digested extract to reach the stabilization time point ($p = 0.912$ between min 45 and 60).

After stabilization of the reaction, rosmarinic acid showed no statistical differences ($p = 0.054$) between the *in vitro* digested sample (RAInt) and the pure compound (RA) (EC₅₀, 60 min = $8.50 \pm 0.33 \mu\text{g/mL}$ and EC₅₀, 45 min = $8.49 \pm 0.59 \mu\text{g/mL}$ respectively).

Again, scavenging activity of the hydroalcoholic extract seems to depend on the pharmaceutical form in which the gastrointestinal digestion was performed. Intestinal absorbable fraction of the extract as powder showed a lower activity ($p < 0.001$) *in vivo* than the encapsulate form at the stabilization point (EC₅₀, 30 min = $30.71 \pm 2.50 \mu\text{g/mL}$ and EC₅₀, 45 min = $21.14 \pm 1.60 \mu\text{g/mL}$, respectively). When compared to the *in vivo* antioxidant activity of the crude extract, the powder form showed no differences ($p = 0.209$, EC₅₀, 45 min = $28.74 \pm 0.58 \mu\text{g/mL}$ before digestion and EC₅₀, 30 min = $30.71 \pm 2.50 \mu\text{g/mL}$ after digestion) but the encapsulated form showed differences with both of them ($p < 0.001$).

Furthermore, there were statistical differences between the samples and the positive control ($p < 0.001$). As expected, empty capsules digested (used as blank for encapsulated extract) and the Blank sample (digestion without sample) did not show any antioxidant activity *in vivo*. For further information, scavenging activity (% inhibition) is compiled in supplementary table S2.

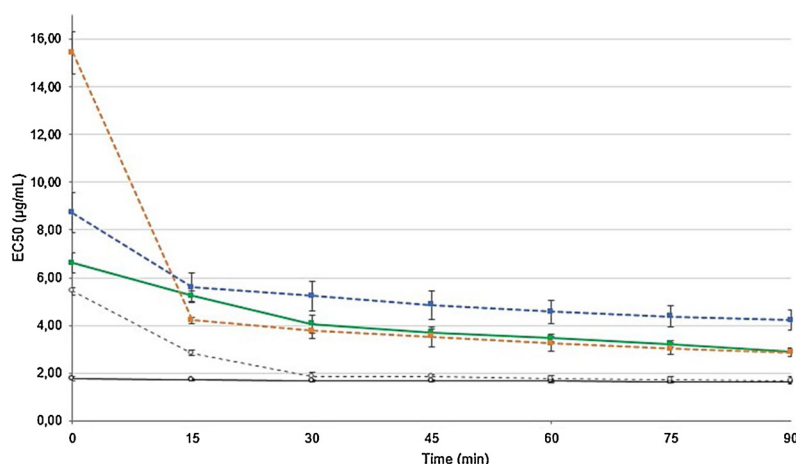
Therefore, the results of *in vitro* and *in vivo* assays to quantify the antioxidant activity pointed that the encapsulated extract shows a higher antioxidant activity than the powder form after the digestion process, but this activity is also greater than the activity of the crude (non-digested) extract (Fig. 5). By contrast, rosmarinic acid used as positive control of antioxidant activity seems to maintain this activity in the *in vitro* and *in vivo* assays after the digestion process.

3.4. Chemical characterisation

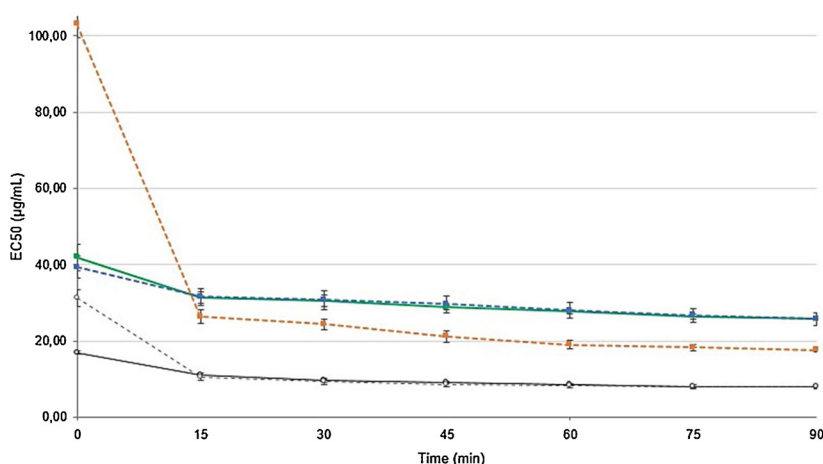
Chemical characterisation of the samples before and after gastrointestinal digestion *in vitro* was performed qualitatively (TLC) and quantitatively (HPLC-UV).

TLC did not show important differences between the two formulations (Fig. 6A). Exposition to NP reagent allowed the identification by colour of the main compounds in the hydroalcoholic extract. Blue is characteristic of phenolic acids (1, 6) and hydroxybenzoic acids (3, 7); yellow-orange is characteristic of flavonoids (2, 4, 5) (Fig. 6A) [19].

Crude hydroalcoholic extract was characterised by HPLC-UV at



	0	15	30	45	60	75	90
—○— RA	1.78 (0.08)	1.71^a (0.04)	1.69 (0.05)	1.67 (0.05)	1.66 (0.05)	1.64 (0.04)	1.63 (0.04)
- -○- - RA Int	5.43 (0.17)	2.84 (0.11)	1.88^a (0.15)	1.84 (0.11)	1.76 (0.12)	1.72 (0.14)	1.69 (0.15)
—●— OV-E	6.62 [*] (0.43)	5.24 (0.22)	4.06^{b,c} (0.39)	3.68 (0.19)	3.47 (0.14)	3.23 (0.15)	2.88 (0.18)
- -●- - OV-C	15.43 (0.91)	4.21 (0.14)	3.77^{b,d} (0.30)	3.52 (0.41)	3.23 (0.31)	3.03 (0.23)	2.86 (0.18)
- -●- - OV-P	8.72 (0.85)	5.59 (0.63)	5.23 (0.64)	4.86^{c,d} (0.58)	4.57 (0.50)	4.37 (0.45)	4.23 (0.43)



	0	15	30	45	60	75	90
—○— RA	16.84 (0.43)	11.03 (0.49)	9.62 (0.65)	8.98 (0.3)	8.50^a (0.33)	8.09 (0.31)	8.04 (0.25)
- -○- - RA Int	31.18 (2.29)	10.34 (0.63)	9.22 (0.56)	8.49^a (0.59)	8.17 (0.58)	7.91 (0.56)	7.88 (0.59)
—●— OV-E	41.87 (3.40)	31.35 (1.41)	30.61 (1.50)	28.74^{b,c} (0.58)	27.68 (0.62)	26.38 (0.70)	25.69 (0.42)
- -●- - OV-C	103.13 (3.48)	26.33 (1.73)	24.34 (1.31)	21.14^{c,d} (1.60)	18.92 (1.04)	18.23 (0.83)	17.59 (0.57)
- -●- - OV-P	39.36 (2.77)	31.59 (2.18)	30.71^{b,d} (2.50)	29.62 (1.84)	28.02 (1.61)	26.71 (1.66)	25.68 (1.23)

325 nm, allowing identification of the seven main compounds by retention time and UV spectrum using standards with the same conditions (Fig. 6B). The AUC of each peak obtained from chromatograms was transformed into concentration by linear regression analysis using known concentrations of the standards to build calibration curves. These standards were caffeic acid ($y = 158,084,586.42x - 137,425.76$; $R^2 = 0.9998$), rosmarinic acid ($y = 38,442,146.87x + 88,581.28$; $R^2 = 1.0000$), apigenin ($y = 301,259,751.23x - 65,541.33$; $R^2 = 0.9998$), luteolin ($y = 117,803,660.17x + 465,907.16$; $R^2 = 0.9999$) and 3, 4-DHBA

($y = 435,588,549.58x - 57,118.73$; $R^2 = 0.9988$).

The amount of the identified compounds was compared among the different samples and expressed in mg of standard per 100 g of sample (Table 1).

As expected, the main compound detected in the hydroalcoholic extract is rosmarinic acid (peak 6; 34.10 ± 0.04 mg in the crude extract) even after the process of digestion for both pharmaceutical forms. This compound has a bioaccessibility of 55.13 % (capsule) and 53.66 % (powder) after digestion. 3,4-dihydroxybenzoic acid (3,4-DHBA; peak

Fig. 3. Temporal evolution of antioxidant activity *in vitro* (the DPPH method) expressed in EC_{50} (mean \pm SD μ g/mL). Continuous lines in the graph correspond to data from non-digested samples and spotted lines to intestinal absorbable samples after digestion. Data for the positive control (rosmarinic acid, RA) are showed in dark grey; green line represents the crude extract (OV-E); dashed orange line represents the intestinal fraction of the two capsules digested (OV-C) and dashed blue line the divided powder after the digestion process (OV-P). The antioxidant activities of the extracts and RA showed differences at all time-points ($p < 0.001$). Table below the graph shows the EC_{50} mean values (SD) expressed in μ g/mL. Bold values indicate the time points in which no differences ($p > 0.05$) with their consecutive value could be observed. ^a and ^b indicate no differences ($p > 0.05$) among the values compared at stabilisation time, whereas ^c and ^d indicate that those values show statistical differences ($p < 0.05$).

Fig. 4. Temporal evolution of antioxidant activity *in vivo* (DPPH method) expressed in EC_{50} (mean \pm SD μ g/mL). Continuous lines in the graph correspond to data from non-digested samples and spotted lines to intestinal absorbable samples after digestion. Data for the positive control (rosmarinic acid, RA) are showed in dark grey; green line represents the crude extract (OV-E); dashed orange line represents the intestinal fraction of the two capsules digested (OV-C) and dashed blue line the divided powder after the digestion process (OV-P). Antioxidant activities of the extracts and RA showed differences in all time-points ($p < 0.001$). Table below the graph shows the EC_{50} mean values (SD) expressed in μ g/mL. Bold values indicate the time points in which no statistical differences ($p > 0.05$) with their consecutive value could be observed. ^a and ^b indicate no statistical differences ($p > 0.05$) among the values compared at stabilisation time, whereas ^c and ^d indicate that those values are statistically differences ($p < 0.05$).

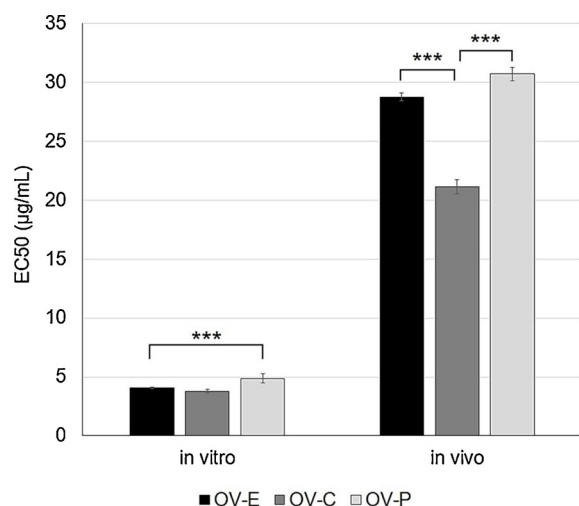


Fig. 5. Antioxidant activity quantified by the DPPH[•] method from the *in vitro* (left) and *in vivo* (right) assays expressed in EC₅₀ (mean and error bars in µg/mL). Values at stabilization point. Black coloured bars represent the crude extract (OV-E); grey bars, the intestinal fraction of the two capsules digested (OV-C) and light grey bars, the powder after digestion process (OV-P). The lower the bar, the more antioxidant the sample is. *** indicates statistical differences between series ($p < 0.001$).

3, 18.04 ± 0.11 mg in the crude extract) is another phenolic acid detected. In this case the bioaccessibility was higher in the powder form (65.85 %) than in the encapsulated form (59.64 %). Luteolin glycoside (peak 4) and the apigenin glycoside (peak 5) are less abundant in the crude extract and their bioaccessibility also depends on the pharmaceutical form (83.31 % and 89.28 % for the encapsulated form and 55.79 % and 54.16 %, respectively, for the powder). The bioaccessibility of caffeic acid (peak 1) is also higher for the encapsulated extract (87.16 % and 77.00 %, for the encapsulated form and powder respectively). The first flavonoid that appears in the chromatogram (luteolin glycoside, peak 2) is potentially bioaccessible in both forms (82.52 % for the encapsulated form and 80.99 % for the powder). Finally, the compound corresponding to peak 7 seems to be a derivate of 3,4-DHBA and it shows a low bioaccessibility in both forms (32.55 % and 24.56 % for the encapsulated form and powder respectively).

In general, both formulations seem to release in the intestine similar but low quantities of phenolic acids (56.79 % for capsules and 54.87 % for powder) and DHBA (43.55 % for capsule and 42.46 % for powder). However, in the case of the flavonoids, quantities depend largely on the formulation (84.96 % for encapsulated form and 63.48 % for powder).

4. Discussion

The pathologies related to aging seem to be related with the unbalance of reactive oxygen species (ROS). The gastrointestinal tract involves ROS as essential signalling intermediates for the maintenance of homeostasis [25] and compounds either able to reduce the unnecessary generation of ROS or neutralize them could be potentially oral treatments to prevent and restore a normal balance. In antioxidant therapies based on herbal preparations, some natural extracts show this activity *in vitro* but lose efficacy when they are tested *in vivo* [26]. This fact may be due to the lack of information on the pharmacokinetics and pharmacodynamics of these extracts [9] but also there are many factors that may influence the stability of their chemical composition. On one hand, it is evident from many studies that the choice of the solvent has a large impact on the stability of the extracts [27,28]. The best stability is acquired when the solvent is ethanol, followed by methanol and DMSO. In fact, the importance of water in the instability of extracts has been largely demonstrated. Water causes redox reactions and the formation of hydroxyl radicals and hydrogen peroxide with oxidative properties. Water also promotes enzymatic reactions, which are especially unfavourable for the extracts in liquid form [29]. Physicochemical properties of the solvents (and temperature) can also affect the chemical composition of the extract and its pharmacological activity [30,31]. Generally, organic solvents tend to extract a greater amount of polyphenols, which are primarily responsible for the antioxidant activity [32]. We chose for this work lyophilized 50 % ethanolic cold extract of *O. vulgare* titrated at 33 % of rosmarinic acid.

The pharmacological actions of *O. vulgare* might be related by its antioxidant activity [1]. For this reason, we have tested the antioxidant activity of an *O. vulgare* extract both *in vitro* and *in vivo* before and after an *in vitro* gastrointestinal digestion process. The interest of this study is that this plant is widely used orally and in this way we could evaluate the effect of gastrointestinal enzymes and the acidity of the stomach on this activity, as this may constitute a key barrier to its effectiveness after oral administration. Moreover, water-based oral medications show a risk of microbiological contamination, may limit the solubility of some important compounds and may require the addition of some excipients that increase costs to achieve agreeable organoleptic characteristics. All this makes liquid oral forms comparatively more expensive, with practical and safety complications in their storage due to their low transportability, large volumes and fragile containers [33,34]. Alternatively, solid oral dosage forms supplement all these disadvantages with greater pharmaceutical and chemical stability, dosing accuracy, better transportability and ease of storage [35].

Among solid oral single-dose pharmaceutical preparations, we have opted for gelatine capsules and divided powder. Encapsulation was chosen due to the simplicity of the preparation of this form that, *a priori*, does not produce any alteration in the bioactive chemical compounds of the extract. Gelatine is a gelling protein widely applied in the food and

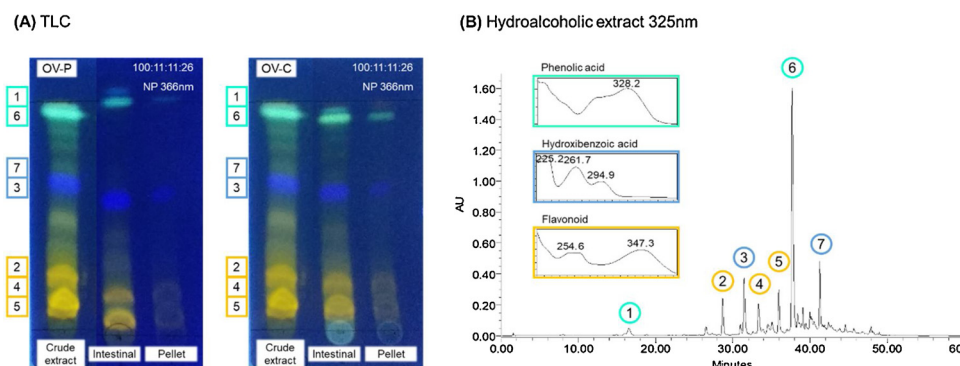


Fig. 6. (A) Thin layer chromatography (TLC) of the two formulations before and after the process of *in vitro* gastrointestinal digestion revealed with NP solution and observed at 366 nm. Left: TLC results corresponding to lyophilised powder. Right: TLC results corresponding to encapsulated extract. In both cases: crude extract corresponds to results of the non-digested hydroalcoholic extract; Intestinal corresponds to the intestinal fraction after digestion and Pellet corresponds to the non-absorbable fraction after digestion. Colour of each compound corresponds to the colour observed on TLC with NP reagent: blue for phenolic acids, green for hydroxybenzoic acid extract before digestion. Main compounds are highlighted with their UV-spectra.

Table 1
Tentative identification of the main compounds detected quantitatively in the hydroalcoholic extract by HPLC-UV, before and after the *in vitro* digestion process. The results for each compound are expressed in mg standard/100 mg dry extract (mean \pm SD) and bioaccessibility (%). Crude extract corresponds to the extract before digestion; encapsulated extract corresponds to data of this extract after digestion; powder is the extract as powder after digestion; Intestinal is the intestinal absorbable fraction; Pellet corresponds to the intestinal non-absorbable fraction. t_R is retention time in minutes, UV-spectra shows the wavelength (in nm) of maximum absorbance. ^a indicates no statistical differences ($p > 0.05$) in the amount of compound between formulations, whereas ^b indicates that there are statistical differences in the amount of compound between formulations ($p < 0.05$).

Type of compound	Peak	Compound	Source	t_R (min)	UV-spectra (nm)	(mg/100 mg dry extract) Bioaccessibility (%)			
						Crude extract		Encapsulated extract	
						Intestinal	Pellet	Intestinal	Pellet
Phenolic acids	1	Caffeic acid	[17,21] and standard	16.58	325	1.87 \pm 0.14	0.20 \pm 0.09	10.69 %	0.24 \pm 0.10
	6	Rosmarinic acid	[17,21] and standard	37.78	329.4	34.10 \pm 0.04	1.63 ^b \pm 0.08	87.16 %	0.6 \pm 0.01
Hydroxybenzoic acids	3	3,4-DHBA	[13] and standard	31.67	225, 261, 294	18.04 \pm 0.11	10.76 ^a \pm 0.07	59.64 %	18.3 ^a \pm 0.01
	7	DHBA derivate	[21,22]	41.21	262, 319	29.15 \pm 0.15	9.49 ^a \pm 0.10	32.55 %	53.66 %
Flavonoids	2	Luteolin glycoside	[21,23] and standard	28.61	254.6, 347.3	7.21 \pm 0.03	1.24 \pm 0.08	0.02 %	11.88 ^a \pm 0.09
	4	Luteolin glycoside	[13,21,23]	33.91	251, 340	7.85 \pm 0.06	5.95 ^a \pm 0.01	82.52 %	65.85 %
	5	Apigenin glycoside	[21,23,24]	36.06	267, 334	7.09 \pm 0.09	6.54 ^b \pm 0.03	83.31 %	27.99 %
							0.91 \pm 0.08	12.62 %	5.84 ^a \pm 0.02
							0.98 \pm 0.04	12.48 %	80.99 %
							0.85 \pm 0.02	11.99 %	55.79 %
							3.84 ^b \pm 0.02	54.16 %	11.85 %
									0.88 \pm 0.03
									12.41 %

pharmaceutical industries. Hard gelatine capsules are easy to swallow, odourless and tasteless, so they mask the unpleasant taste of products. However, sometimes they have some hygroscopic problems (the capsules contain around 12 % water) so we added silicon dioxide as anti-agglomerant to facilitate its industrial production, drug release and digestion. As for its size, they are only physical containers and the small capsules limit their content and the large ones are difficult to swallow so we choose number 2 with a capacity of 0.36 mL.

The second formulation was powder, which is not physically limited in dosage. The powder can be presented as a unidose (divided powder) or multidose (bulk powder). Unidose powder preparations are ready-to-take whereas the bulk powders may need a dispenser device (like a measuring spoon). Regardless of the packaging, the powder consists of solid, loose and dry particles designed to be resuspended in water avoiding the inconvenience of swallowing for some patients with problems of this type. On the other hand, depending on the type of compound this formulation may need coloring and / or flavoring substances. Thus, like capsules, it can contain one or more active ingredients, with or without excipients. In this case we selected silicon dioxide for the same reasons as before.

Despite the fact that *O. vulgare* is widely used in traditional medicine, there is no a formal monography that compiles recommendations for therapeutic doses. European Medicine Agency (EMA) has two published monographies for two close subspecies: *O. majorana* and *O. dictamnus*. Our hydroalcoholic extract showed a yield of 28.48 %, which means that 1.13 g of extract is comparable to 4 g of dried plant. According to EMA indications, daily dose for *O. majorana* is between 2 and 4 g of dried plant, once or twice after meals [36]. Thus, as an orientation for a therapeutic dose of *O. vulgare* we selected the lowest daily dose, that is, 509 mg of extract. According to the size capsule chart, the capsules number 2 (with 0.36 mL of capacity) admit up to 250 mg of extract and 0.1 mg of excipient. Therefore, two capsules or 500 mg of the divided powder could be considered as the daily dose.

Uniformity and mass content assays were performed for all samples according to the *Pharmacopeia* (Real Farmacopea Española 5th ed., 2006, [37]), being 61.14 ± 0.34 mg for individual empty capsules (122.29 ± 0.18 mg for daily dose), 311.15 ± 0.94 mg for individual full-filled capsules (with a content of 250.01 ± 0.64 mg) and 502.04 ± 0.06 mg for the powder. Besides, the disintegration assay for the capsules (empty and fulfilled) was provided by the *in vitro* gastrointestinal digestion process. The powder showed solubility in water at 40 mg/mL, so the daily dose can be easily administered dissolved in a glass of water. Both formulations showed chemical *ex corpore* characteristics suitable to be administered in an *in vivo* model.

Drug release is the first crucial step in pharmacokinetics (LADME system) in which small differences could explain variations in the pharmacological activity of the preparation. As shown (Fig. 2), the antioxidant activity *in vitro* of the extract depends on the formulation. Encapsulated extract is able to maintain this activity after an *in vitro* gastrointestinal digestion process, assuming that the capsule protects the extract (with an EC_{50} , 30 min = 4.06 ± 0.39 μ g/mL before digestion and an EC_{50} , 30 min = 3.77 ± 0.30 μ g/mL after digestion; $p = 0.981$). Thus, the capsule could preserve the activity by protecting the compounds that, in the case of dry powder, are directly exposed to stomach acidity and enzymes (pepsin) (EC_{50} , 45 min = 4.86 ± 0.58 μ g/mL, $p < 0.001$). On the other hand, the capsule disintegration itself seems to make the extract reach its maximum activity 15 min later than the powder or rosmarinic acid. Finally, the encapsulated extract achieves stability in its activity in a similar manner to the non-digested sample (at min 30), perhaps due to less chemical and structural damage suffered by its components. Empty-digested capsules showed a baseline scavenging activity, so they did not show antioxidant activity (supplementary table S1).

The total bioaccessibility was similar for both formulations, being 87.17 % for the capsules and 83.11 % for the powder. Metabolism and absorption usually depend on the physicochemical characteristics of the

phenolic compounds (basic structure, molecular size, degree of polymerization or glycosylation, solubility and conjugation with other phenolic compounds) [16]. HPLC-UV analysis (Table 2) helped us to check the evolution of the compounds in the extract after the digestion process. Phenolic acids seem to be affected by this process because their total amount for intestinal absorption was around 50 % for both formulations. Nevertheless, the capsules seem to have protected flavonoids more, since their bioaccessibility was 84.96 % for the encapsulated form and 63.48 % for the powder. The two flavonoids (apigenin glycoside and luteolin glycoside) and caffeic acid, all of them known antioxidants, were significantly less present in the intestinal fraction of the powder and could be the compounds that explain the different bioactivities of both formulations. These compounds were also found in the non-absorbable fraction of both formulations, explaining the yellow spots of the qualitative antioxidant assay by TLC (Fig. 2). The bioaccessibilities of four of the seven identified compounds were not formulation-dependent and they were minimally found in pellets (intestinal non-absorbable fractions).

Nonetheless, both formulations were shown to have potential antioxidant activity *in vitro* due to the chemical stability of the components of the extracts. For example, bioaccessibility of rosmarinic acid (considered the primary constituent in both formulations) was 55.1 % in the capsule but also 53.6 % in the powder. In fact, we used rosmarinic acid as positive control as it maintained its antioxidant activity *in vitro* before and after digestion when administered as a powder.

C. elegans is a simple *in vivo* model widely used for *in vivo* screenings and functional analyses. Although antioxidant activity in this organism can be assayed by quantifying in parallel the protein and gene expression [38] we have used a simple and reliable method recently developed [12] to quantify the internal antioxidant activity. With this assay, we have verified that the antioxidant activity of the different samples previously analysed *in vitro* is preserved in this organism. Thus, rosmarinic acid (the main component of the *O. vulgare* extracts) also behaves as antioxidant before and after the digestion process (EC_{50} , 60 min = $8.50 \pm 0.33 \mu\text{g/mL}$ and EC_{50} , 45 min = $8.49 \pm 0.59 \mu\text{g/mL}$; $p = 0.054$, respectively). As expected, in worms, the encapsulated extract showed also a higher antioxidant activity than the powder (EC_{50} , 45 min = $21.14 \pm 1.60 \mu\text{g/mL}$ and EC_{50} , 30 min = $30.71 \pm 2.50 \mu\text{g/mL}$, respectively with $p < 0.001$), probably due to the variation in the amount of flavonoids [39]. In fact, the non-digested extract showed also a higher antioxidant activity *in vivo*. However, and surprisingly, the digested encapsulated extract is more antioxidant than the pure non-digested extract (Fig. 5). In addition, there is an analogy between the results obtained in the *in vitro* and *in vivo* assays although the differences are increased in the *in vivo* assays.

The crude extract was not digested, so we expected a similar behaviour to that of the encapsulated extract. When we analysed the antioxidant activity *in vitro*, the crude extract showed maximum activity but *in vivo* assays require exposure of the sample to the physiological conditions of the worm. For example, although empty capsules did not show antioxidant activity *in vitro*, their activity *in vivo* increased up to 39.15 ± 9.00 % displaying a similar behaviour to glucose [12]. Thus, the baseline data obtained in the *in vivo* assays could be associated to antioxidative pathways activated in the worms [40].

The quantification of antioxidant activity over time allows the analysis of its stability. The reaction is fast when stability (maximum activity) is reached before 30 min, it is intermediate when it is reached between 30 and 45 min, and it is slow when it is reached after 60 min. Thus, rosmarinic acid after digestion is an intermediate antioxidant both *in vitro* and *in vivo* (Figs. 3 and 4, *in vitro* ≥ 30 min and *in vivo* ≥ 45 min), while before digestion, it is a fast antioxidant *in vitro* and a slow antioxidant *in vivo* (the maximum points of antioxidant activity were at 15 and 60 min, respectively). Establishing the crude non-digested extract as the control for the kinetic analyses, the digested powder behaves similarly to rosmarinic acid reaching a stable time point 15 min later *in vitro* and 15 min earlier *in vivo*. On the contrary,

the encapsulated form behaves similarly to the digested rosmarinic acid reaching stability after 30 min *in vitro* and after 45 min *in vivo*.

In any case, according to the kinetic classification, there are no differences between samples. The hydroalcoholic extract is an intermediate antioxidant before and after digestion both *in vitro* and *in vivo* for the two formulations. In this sense, the stability of the reaction does not have an important role in the differences observed between the digested powder and the encapsulated extract. However, the encapsulated form always shows a higher activity, so we consider that it could be the optimal oral formulation for *O. vulgare* hydroalcoholic extracts.

5. Conclusions

In conclusion, the oral pharmaceutical form may have an important role in the pharmacological activity also for medicinal plant extracts. Compared to powder, the encapsulation can protect physically the components of the extracts from the digestion process, favouring the maintenance of their antioxidant activity. Thus, two capsules of 250 mg of a hydroalcoholic extract of *O. vulgare* (with a minimum of 33 % of rosmarinic acid) could be a potentially minimum daily dose for antioxidant oral treatment.

Author contributions

M.P.d.T, M.I.C., and J.L.V. conceived the experimental design and wrote the paper. M.P.d.T. performed *in vitro* and *in vivo* experiments. M.P.d.T. designed and performed statistical analyses. M.P.d.T, R.Y.C., M.I.C., and J.L.V. critically reviewed the data contributing to the draft of the manuscript.

Funding

We are grateful to the PIUNA (Plan Investigador de la Universidad de Navarra, Ref. PIUNA/2018) and the Ministerio de Economía y Competitividad – Spain (AGL2014-52636-P) for the financial support of this work.

Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2020.110424>.

References

- [1] S. Akerreta, M.I. Calvo, R.Y. Caverio Remón, Sabiduría popular y plantas curativas: recopilación extraída de un estudio etnobotánico en Navarra, 1st ed., (2013), pp. 232–233 Ediciones I, Madrid.
- [2] B. Teixeira, A. Marques, C. Ramos, C. Serrano, O. Matos, N.R. Neng, J.M.F. Nogueira, J.A. Saraiva, M.L. Nunes, Chemical composition and bioactivity of different oregano (*Origanum vulgare*) extracts and essential oil, J. Sci. Food Agric. 93 (2013) 2707–2714, <https://doi.org/10.1002/jsfa.6089>.
- [3] M. Shokrzadeh, A. Ahmadi, A. Chabra, F. Naghshvar, F. Salehi, E. Habibi, H. Haghi-Aminjan, An ethanol extract of *Origanum vulgare* attenuates cyclophosphamide-induced pulmonary injury and oxidative lung damage in mice, Pharm. Biol. 52 (2014) 1229–1236, <https://doi.org/10.3109/13880209.2013.879908>.
- [4] C. Sarikurkcu, G. Zengin, M. Oskay, S. Uysal, R. Ceylan, A. Aktumsek, Composition, antioxidant, antimicrobial and enzyme inhibition activities of two *Origanum vulgare* subspecies (subsp. *vulgare* and subsp. *hirtum*) essential oils, Ind. Crops Prod. 70 (2015) 178–184, <https://doi.org/10.1016/j.indcrop.2015.03.030>.
- [5] G. Zengin, C. Ferrante, G. Orlando, D. Zheleva-Dimitrova, R. Gevrenova, L. Recinella, A. Chiavaroli, S. Leone, L. Brunetti, M.Z. Aumeeruddy, A. Aktumsek, M.F. Mahomoodally, P. Angelini, S. Covino, R. Venanzoni, B. Tirillini, L. Menghini, Chemical profiling and pharmacotoxicological activity of *Origanum sipyleum* extracts: exploring for novel sources for potential therapeutic agents, J. Food Biochem. 43 (2019), <https://doi.org/10.1111/jfbc.13003>.

- [6] S. Arami, A. Ahmadi, S.A. Haeri, The radioprotective effects of *Origanum vulgare* extract against genotoxicity induced by 131 I in human blood lymphocyte, *Cancer biother. Radiopharm.* 28 (2013) 201–206, <https://doi.org/10.1089/cbr.2012.1284>.
- [7] E. Habibi, M. Shokrzadeh, A. Ahmadi, A. Chabra, F. Naghshvar, R. Keshavarz-Maleki, Genoprotective effects of *Origanum vulgare* ethanolic extract against cyclophosphamide-induced genotoxicity in mouse bone marrow cells, *Pharm. Biol.* 53 (2015) 92–97, <https://doi.org/10.3109/13880209.2014.910674>.
- [8] E. Habibi, M. Shokrzadeh, A. Chabra, F. Naghshvar, R. Keshavarz-Maleki, A. Ahmadi, Protective effects of *Origanum vulgare* ethanol extract against cyclophosphamide-induced liver toxicity in mice, *Pharm. Biol.* 53 (2015) 10–15, <https://doi.org/10.3109/13880209.2014.908399>.
- [9] R.N. Alolga, Y. Fan, G. Zhang, J. Li, Y.-J. Zhao, J. Lelu Kakila, Y. Chen, P. Li, L.-W. Qi, Pharmacokinetics of a multicomponent herbal preparation in healthy Chinese and African volunteers, *Sci. Rep.* 5 (2015) 12961, <https://doi.org/10.1038/srep12961>.
- [10] M. Nur Alam, N. Jahan Bristi, M. Rafiquzzaman, Review on in Vivo and in Vitro Methods Evaluation of Antioxidant Activity, (2013), <https://doi.org/10.1016/j.jsps.2012.05.002>.
- [11] K. Koch, S. Havermann, C. Büchter, W. Wätjen, *Caenorhabditis elegans* as model system in pharmacology and toxicology: effects of flavonoids on redox-sensitive signalling pathways and ageing, *Scientific World J.* 2014 (2014) 920398, <https://doi.org/10.1155/2014/920398>.
- [12] M.P. de Torre, R.Y. Caverio, M.I. Calvo, J.L. Vizmanos, A simple and a reliable method to quantify antioxidant activity in vivo, *Antioxidants* (2019), <https://doi.org/10.3390/antiox8050142>.
- [13] L. Gayoso, M. Roxo, R.Y. Caverio, M.I. Calvo, D. Ansorena, I. Astiasarán, M. Wink, Bioaccessibility and biological activity of *Melissa officinalis*, *Lavandula latifolia* and *Origanum vulgare* extracts: influence of an in vitro gastrointestinal digestion, *J. Funct. Foods* 44 (2018) 146–154, <https://doi.org/10.1016/j.jff.2018.03.003>.
- [14] M. Alming, A.-M. Aura, T. Bohn, C. Dufour, S.N. El, A. Gomes, S. Karakaya, M.C. Martínez-Cuesta, G.J. McDougall, T. Requena, C.N. Santos, In vitro models for studying secondary plant metabolite digestion and bioaccessibility, *Compr. Rev. Food Sci. Food Saf.* 13 (2014) 413–436, <https://doi.org/10.1111/1541-4337.12081>.
- [15] D.H. Jornada, G.F. Dos Santos Fernandes, D.E. Chiba, T.R.F. De Melo, J.L. Dos Santos, M.C. Chung, The prodrug approach: a successful tool for improving drug solubility, *Molecules* 21 (2016), <https://doi.org/10.3390/molecules21010042>.
- [16] J.M. Carbonell-Capella, M. Buniowska, F.J. Barba, M.J. Esteve, A. Frígola, Analytical methods for determining bioavailability and bioaccessibility of bioactive compounds from fruits and vegetables: a review, *Compr. Rev. Food Sci. Food Saf.* 13 (2014) 155–171, <https://doi.org/10.1111/1541-4337.12049>.
- [17] L. Gayoso, A.S. Claerbout, M.I. Calvo, R.Y. Caverio, I. Astiasarán, D. Ansorena, Bioaccessibility of rutin, caffeic acid and rosmarinic acid: influence of the in vitro gastrointestinal digestion models, *J. Funct. Foods* 26 (2016) 428–438, <https://doi.org/10.1016/j.jff.2016.08.003>.
- [18] W. Brand-Williams, M.E. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity, *LWT - Food Sci. Technol.* 28 (1995) 25–30, [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5).
- [19] H. Wagner, S. Bladt, *Plant Drug Analysis*, 2nd ed., Springer (ed.), Germany, 1996.
- [20] R. Pinacho, R.Y. Caverio, I. Astiasarán, D. Ansorena, M.I. Calvo, Phenolic compounds of blackthorn (*Prunus spinosa* L.) and influence of in vitro digestion on their antioxidant capacity, *J. Funct. Foods* 19 (2015) 49–62, <https://doi.org/10.1016/J.JFF.2015.09.015>.
- [21] M.D. González, C.M. Luis, P.L. Lanzelotti, Polyphenolic profile of *Origanum vulgare* L. Spp. *Viridulum* from Argentina, *Fyt. Issn.* 9457 (2014) 179–184 <http://www.scielo.org.ar/pdf/phyton/v83n1/v83n1a23.pdf>.
- [22] C.H. Liang, L.P. Chan, H.Y. Ding, E.C. So, R.J. Lin, H.M. Wang, Y.G. Chen, T.H. Chou, Free radical scavenging activity of 4-(3,4-Dihydroxybenzoyloxymethyl) phenyl- O-β-D-glucopyranoside from *origanum vulgare* and its protection against oxidative damage, *J. Agric. Food Chem.* 60 (2012) 7690–7696, <https://doi.org/10.1021/jf302329m>.
- [23] S. Đorđević, M. Kakić, S. Amr, the Extraction of Apigenin and Luteolin From the Sage *Salvia officinalis* L. From Jordan, *Work. Living Environ. Prot.* 1 (2000) 87–93.
- [24] L.Z. Lin, S. Mukhopadhyay, R.J. Robbins, J.M. Harnly, Identification and quantification of flavonoids of Mexican oregano (*Lippia graveolens*) by LC-DAD-ESI/MS analysis, *J. Food Anal.* 20 (2007) 361–369, <https://doi.org/10.1016/j.jfca.2006.09.005>.
- [25] G. Aviello, U.G. Knaus, NADPH oxidases and ROS signaling in the gastrointestinal tract review-article, *Mucosal Immunol.* 11 (2018) 1011–1023, <https://doi.org/10.1038/s41385-018-0021-8>.
- [26] O. Firuzi, R. Miri, M. Tavakkoli, L. Saso, Antioxidant therapy: current status and future prospects, *Curr. Med. Chem.* 18 (2012) 3871–3888, <https://doi.org/10.2174/092986711803414368>.
- [27] V.J. Barwick, Strategies for solvent selection - a literature review, *TrAC - Trends Anal. Chem.* 16 (1997) 293–309, [https://doi.org/10.1016/S0165-9936\(97\)00039-3](https://doi.org/10.1016/S0165-9936(97)00039-3).
- [28] A. Nn, A review on the extraction methods use in medicinal plants, principle, strength and limitation, *Med. Aromat. Plants.* 04 (2015) 3–8, <https://doi.org/10.4172/2167-0412.1000196>.
- [29] S. Gafner, C. Bergeron, The challenges of chemical stability testing of herbal EXtracts in finished products using state-of-the-Art analytical methodologies, *Curr. Pharm. Anal.* 1 (2005) 203–215.
- [30] L. Tomsone, Z. Kruma, Comparison of different solvents for isolation of phenolic compounds from horseradish (*Armoracia rusticana* L.) leaves, *Res. Rural Dev.* 1 (2013) 104–110.
- [31] D.A.J.C. Arrier, Comparison of the chemical composition of extracts from *Scutellaria lateriflora* using accelerated solvent extraction and supercritical fluid extraction versus standard hot water or 70 % ethanol extraction, *J. Agric. Food Chem.* (2005) 3076–3080, <https://doi.org/10.1021/jf048408t>.
- [32] M.F. Mahomoodally, G. Zengin, M.O. Aladag, H. Ozparlak, A. Diuzheva, J. Jekő, Z. Cziák, M.Z. Aumeeruddy, HPLC-MS/MS chemical characterization and biological properties of *Origanum onites* extracts: a recent insight, *Int. J. Environ. Health Res.* 29 (2019) 607–621, <https://doi.org/10.1080/09603123.2018.1558184>.
- [33] A. Lajoie, E. Henin, B. Kassai, D. Terry, Solid oral forms availability in children: a cost saving investigation, *Br. J. Clin. Pharmacol.* 78 (2014) 1080–1089, <https://doi.org/10.1111/bcp.12442>.
- [34] R.G. Strickley, Solubilizing excipients in oral and injectable formulations, *Pharm. Res.* 21 (2004) 201–230, <https://doi.org/10.1023/B:PHAM.0000016235.32639.23>.
- [35] Y. Qiu, Y. Chen, G.G.Z. Zhang, L. Yu, R.V. Mantri, *Developing Solid Oral Dosage Forms: Pharmaceutical Theory and Practice*, second edition, Elsevier Inc., 2016.
- [36] E. Medicines Agency, European Union Herbal Monograph on *Origanum majorana* L., *Herba*, (2016) (Accessed March 14, 2018), http://www.ema.europa.eu/docs/en_GB/document_library/Herbal_Herbal_monograph/2016/11/WC500216168.pdf.
- [37] *Real Farmacopea Española*, Publicada por El Ministerio De Sanidad y Consumo, por Mandato de la Ley 25/1990, de 20 de diciembre, del Medicamento, 5th ed., Ministerio de Sanidad y Consumo: Boletín Oficial del Estado, Madrid, 2015.
- [38] R. Doonan, D. Gems, Interpreting Intervention Studies Antioxidant defense and aging in *C. elegans* Is the oxidative damage theory of aging wrong? (2009) (Accessed January 14, 2019), <http://www.landesbioscience.com/journals/cc/article/8595>.
- [39] M.H.H. Roby, M.A. Sarhan, K.A.H. Selim, K.I. Khalel, Evaluation of antioxidant activity, total phenols and phenolic compounds in thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.), and marjoram (*Origanum majorana* L.) extracts, *Ind. Crops Prod.* 43 (2013) 827–831, <https://doi.org/10.1016/j.indcrop.2012.08.029>.
- [40] D.B. Magner, J. Wollam, Y. Shen, C. Hoppe, D. Li, C. Latza, V. Rottiers, H. Hutter, A. Antebi, The NHR-8 nuclear receptor regulates cholesterol and bile acid homeostasis in *C. Elegans*, *Cell Metab.* 18 (2013) 212–224, <https://doi.org/10.1016/j.cmet.2013.07.007>.